Research Paper

Eurycomanone Induces Apoptosis through the Up-Regulation of p53 in Human Cervical Carcinoma Cells

Nurkhasanah Mahfudh[1] and Azimahtol Hawariah Lope Pihie

School of Biosciences and Biotechnology, National University of Malaysia, 43600 Bangi, Selangor, Malaysia [N. Mahfudh, A. H. Lope Pihie]; Faculty of Pharmacy Ahmad Dahlan University, Jl. Prof Soepomo, Janturan, Yogyakarta, Indonesia [N. Mahfudh]

AIM: **Eurycomanone is a cytotoxic compound found in *Eurycoma longifolia* Jack. Previous studies had noted the cytotoxic effect against various cancer cell lines. The aim of this study is to investigate the cytotoxicity against human cervical carcinoma cells and the mode of action.**

METHODS: **The cytotoxicity of eurycomanone was evaluated using methylene blue staining assay and the mode of cell death was detected by Hoechst 33258 nuclear staining, TUNEL assay and flow cytometry with Annexin-V/propidium iodide double staining. The protein expression of p53, E6, E6-AP, Bax and Bcl-2 were studied by Western blotting. Immunostaining assay was used to confirm the up-regulation of p53 and Bax in cancer cells.**

RESULTS: **The findings suggested that eurycomanone was cytotoxic on cancerous cells (CaOv-3, HeLa, HepG2, HM3KO, MCF-7) and less toxic on normal cells (MDBK, Vero). Furthermore, various methods proved that apoptosis was the mode of death in eurycomanone-treated cervical cancer HeLa cells. The characteristics of apoptosis including chromatin condensation, DNA fragmentation and apoptotic bodies were found following eurycomanone treatment. This study also found that apoptotic process triggered by eurycomanone involved the up-regulation of p53 tumor suppressor protein. The up-regulation of p53 was followed by the increasing of pro-apoptotic Bax and decreasing of anti-apoptotic Bcl-2. However, eurycomanone did not affect the E6 and E6-AP protein expression.**

CONCLUSION: **The data suggest that eurycomanone was cytotoxic on HeLa cells by inducing apoptosis through the up-regulation of p53 and Bax, and down-regulation of Bcl-2, independently of functional E6 and E6-AP activity.**

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Keywords:

eurycomanone

apoptosis

p53

Bax

Introduction

Cervical cancer is an important health problem worldwide, being the second most common cancer among women and first ranking in many developing countries [1]. In Malaysia, cancer of the cervix was the second most common cancer among women. The National Cancer Registry in 2004 reported the incidence of cervical cancer was 12.9% of total female cancers. There were a total of 1,557 cases of cancer cervix, with an ASR of 19.7 per 100,000 populations [2].

Specific types of human papillomavirus (HPV[2]), mainly types 16 and 18, have been identified as causative agents of at least 90% cervical cancer and are also linked to more than 50% of other anogenital cancers [3,4]. Cervical carcinoma is initiated by infection with a high risk HPV, usually HPV type 16 (HPV16) or HPV18, and gene transfer studies have identified the E6 and E7 genes as the major HPV oncogenes [5].

The expression of p53 is very low in cervical cancer cells [6]. The E6 and the E7 proteins modulate cellular proteins that regulate the cell cycle [7,8]. In cooperation with the cellular proteins E6-AP, the E6 protein binds to the tumor suppressor protein p53, and targets it for accelerated degradation. However, in response to DNA damage stress, p53 is stabilized and induced to exert its various cellular responses including apoptosis [9,10].

Eurycoma longifolia Jack is one of the popular folk medicines of South East Asia including Myanmar, Indochina, Thailand, Laos, Cambodia and Malaysia [11]. *E. longifolia* is identified locally as Tongkat Ali in Malaysia, Pasakbumi or Bidara Pahit in Indonesia, lan-don in Thailand and "Cay ba binh" in Vietnam translated as a tree which cures hundreds of diseases [12].

Several classes of compounds have been isolated and identified, including quassinoids [12-22], canthin-6-one alkaloids [12,23], β-carboline alkaloids [11,12], triterpene tirucallane type [18], squalene derivatives [24] and biphenylneolignan [25].

Previous study of methanolic extract showed induction of apoptosis in human breast cancer MCF-7 cell lines via decreasing of Bcl-2 expression [26]. Eurycomanone isolated

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[1]**Correspondence:** Dr. Nurkhasanah Mahfudh, School of Biosciences and Biotechnology, National University of Malaysia, 43600 Bangi, Selangor, Malaysia. E-mail: nurkhas@gmail.com

[2]**Abbreviations:** HPV, human papillomavirus; TUNEL, TdT-mediated dUTP nick end labeling; FITC, fluorescein isothiocyanate; PI, propidium iodide; PS, phosphatidylserine.

from *E. longifolia* was also proven to inhibit the growth of MCF-7 cells by triggering apoptosis through down-regulation of the anti-apoptotic protein Bcl-2. It is relatively non-toxic in non-cancerous breast cell lines (MCF-10A)[27].

The objective of this study is to evaluate the anti-proliferative effect of eurycomanone and the mechanism of cell death in human cervical carcinoma HeLa cells.

Materials and Methods

Compound extraction

Eurycomanone (Figure 1) was extracted from the root of *E. longifolia* as previously described [13]. The root of the plant (1350 g) was dried, ground and extracted with methanol. The methanol extract was then concentrated to dryness. A suspension of the dry extract (500 g) in water was separated with diethyl ether using separating funnel. The water fraction was then separated with n-buthanol saturated with water. The buthanol layer was then evaporated to dryness, and the dry buthanol fraction (20 g) was subjected to column chromatography over silica gel using a mixture of ethyl acetate-ethanol-water (100:10:1) as the mobile phase. The white crystal (0.16 g) was found and identified as eurycomanone by comparison of NMR spectral data with published value [12,28].

Cell culture

All cell lines (CaOv-3, HeLa, HepG2, HM3KO, MCF-7, Vero, and MDBK) were obtained from American Type Culture Collection (Rockville, MD, USA). The cell lines were maintained in DMEM medium supplemented with 5% FBS and 1% penicillin-streptomycin. The cells were grown at 37°C in humidified atmosphere of 5% CO_2.

Cell proliferation assay

The anti-proliferative effect of eurycomanone on cancer cells was evaluated by determining the IC_{50} values for the above mentioned cell lines as previously described [29]. Logarithmically growing cells were seeded at a density of 5 × 10^3 cells/well into 96-well plates and allowed to adhere for 24 h at 37°C. Then the seeding medium was discarded and replaced with fresh medium containing varying concentrations of eurycomanone. The cells were maintained for 3 days and the anti-proliferative activity of eurycomanone was determined using the methylene blue staining. Glutaraldehyde was added to each well to a final concentration of 2.5% (v/v). After 15-min incubation, the cells were washed with 0.15 M NaCl. The cells were then stained with 0.1 ml of 0.05% methylene blue solution for 15 min. The excess dye was washed out and 0.2 ml of 0.33 M HCl was added into each well. The absorbance was read at 660 nm.

TUNEL assay

DNA fragmentation, one of the characteristics of apoptotic cells, was examined by TdT-mediated dUTP nick end labeling (TUNEL) assay. HeLa cells were seeded onto a poly-L-lysine slide in a petri dish. After 24 h adherence, the cells were incubated without or with eurycomanone (5 μM) for 24 and 72 h. Tamoxifen (21.5 μM) was used as positive control. At the end of the treatment, the cells were fixed with 4% methanol-free paraformaldehyde in PBS at 4°C for 30 min and then washed with PBS for further analysis. The cells were stained with fluorescent TUNEL assay kit (Promega, Madison, WI, USA) according to the manufacturer's instruction. This assay detects apoptotic cells when visualized through the fluorescence microscope (Leica, Vertrieb Deutschland, Solms, Germany).

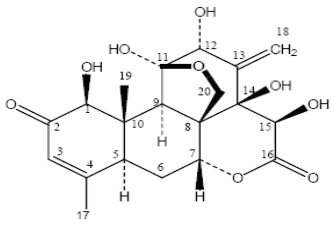

Figure 1: Chemical structure of eurycomanone

Nuclear staining assay

Staining with Hoechst 33258 was performed according to the method previously described [30]. HeLa cells were treated with 5 μM of eurycomanone for 24 h, and then the cells were washed with PBS and fixed with 4% methanol-free paraformaldehyde for 30 min at 4°C. The fixed cells were washed with PBS and stained with Hoechst 33258 (Sigma, St. Louis, MO, USA) at a final concentration of 30 μg/ml. The slides were observed under fluorescence microscope (Leica).

Flow cytometry analysis

The number of apoptotic cell death induced by eurycomanone was measured by flow cytometry using the APOPTEST™-FITC kit according to manufacturer's protocol (DakoCytomation, Glostrup, Denmark). The treated and untreated HeLa cells were harvested and washed with cold PBS. An aliquot (10^5 cells/100 μl) of cell suspension was added with 1 μl fluorescein isothiocyanate (FITC)-conjugated annexin-V and 2.5 μl propidium iodide (PI; 250 μg/ml). After 10-min incubation on ice, the cells were measured immediately.

Western blotting

Aliquots (20 μg) of protein extracts from treated and untreated HeLa cells were separated on 12% SDS-polyacrylamide gels. After electrophoresis, the proteins were blotted onto polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat milk in PBS plus 0.1% Tween-20, then incubated with primary antibodies for p53, E6, E6-AP, Bcl-2 and Bax (BD Biosciences, San Jose, CA, USA) diluted at 1:1000, and detected with horseradish peroxidase-conjugated antibodies. Protein band were detected by ECL system (Amersham Biosciences, Piscataway, NJ, USA). The membranes were re-probed with β-actin as an internal control. Relative band intensities were determined by quantitation of each band with an image analyzer (Alpha-InnoTech, Avery Dennison, CA, USA).

Immunostaining

HeLa cells were fixed on slides and permeabilized with Triton X-100 for 20 min at 4°C and blocked with 2% FBS in PBS for 2 h at 37°C. After washing with PBS, the cells were incubated with anti-p53 antibody (BD Biosciences) overnight at a dilution of 1:250 at 4°C. Following incubation, the slides were washed and incubated with FITC-conjugated secondary antibody. After washing, the slides were visualized with fluorescence microscope (Leica).

Results

Effect of eurycomanone on cell viability

Eurycomanone significantly reduced the viability and proliferation of cancer cells (CaOv-3, HeLa, HepG2, HM3KO and MCF-7) in a concentration-dependent manner (Figure 2). The IC_{50} values of eurycomanone on cancer cell lines were found around 5-10 μM with the IC_{50} on HeLa cells was the lowest. Eurycomanone was also found to be relatively non-

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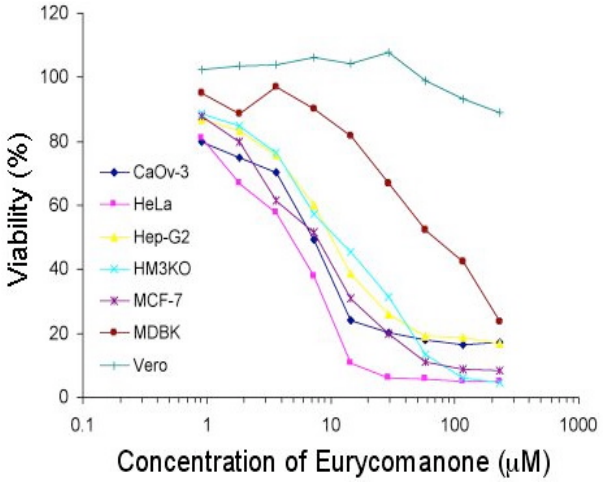

Figure 2: Cytotoxic effect of eurycomanone on cancer cells. Treatment of eurycomanone on cancerous cells (CaOv-3, HeLa, HepG2, HM3KO and MCF-7) significantly reduced the number of viable cell. It was relatively non-toxic against non-cancerous cells (MDBK, Vero). Results were represented as means of at least three independent experiments.

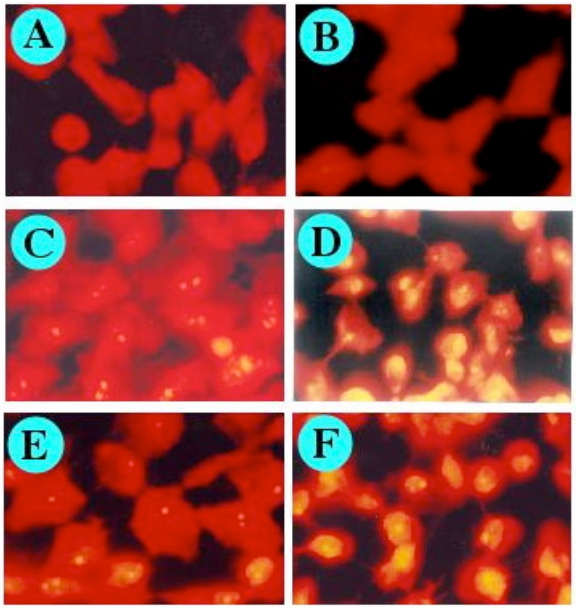

Figure 3: TUNEL assay of HeLa cells. In untreated HeLa cells (A) or HeLa cells treated with DMSO (B), no obvious fluorescence was detected in the nuclei, as the cells did not exhibit significant DNA fragmentation. HeLa cells treated with eurycomanone at 24 h (C) or 72 h (D), as well as HeLa cells treated with tamoxifen at 24 h (E) or 72 h (F) showed fluorescence in the nuclear regions, indicating occurrence of DNA fragmentation. The fluorescence became more intense with longer treatment duration.

toxic to normal cells (MDBK and Vero). These findings suggested the potential of eurycomanone as anti-cancer agent.

Eurycomanone-induced apoptotic cell death

The mode of killing that is induced by most anti-cancer agents is by apoptotic cell death. The anti-proliferative activity shown by eurycomanone could be possibly due to the induction of apoptosis. To determine the mode of growth inhibition in HeLa cells induced by eurycomanone, TUNEL assay and nuclear staining with Hoechst 33258 were carried out. HeLa cells were treated with 5 μM eurycomanone for 24 or 72 h. Tamoxifen was used as positive control while the negative control was treated with DMSO. After treated with eurycomanone for 24 h, HeLa cells showed active apoptosis and the fragmented DNA were labeled with fluorescence in the nuclei by TUNEL assay (Figure 3C). At 72 h, more fluorescence was detected, indicating that more cells were undergoing apoptosis (Figure 3D). The positive control of tamoxifen displayed similar nuclear fluorescence, indicating TUNEL-positive apoptotic cells (Figure 3E and 3F). In HeLa cells treated with DMSO as negative control, no fluorescence was detected in the nuclei, due to the absence of fragmented DNA (Figure 3B). This result was also displayed from untreated HeLa cells (Figure 3A). The cell undergoing apoptosis display a profound destruction of the nucleus that results in the formation of nuclear blebs containing DNA. Staining of apoptotic cells with a fluorescent DNA-binding dye allows for easy detection of this phenomenon [31]. The morphology of apoptotic cells, including chromatin condensation and formation of apoptotic bodies, can be determined after staining with Hoechst 33258 that binds at specific sites of double-stranded DNA and displays a green fluorescence. Hoechst 33258 stains both healthy and apoptotic cells, however, healthy cells will stain homogenously while apoptotic cells will stain brightly [32]. Staining with Hoechst 33258 on the HeLa cells treated with eurycomanone for 24 h showed intense fluorescence in the nuclei (Figure 4B), thereby indicating chromatin condensation. At 72 h, HeLa cells furthermore exhibited apoptotic bodies (Figure 4C).

Next, flow cytometry analysis was used to quantify eurycomanone-induced apoptotic cells by measuring the externalization of phosphatidylserine (PS). In many cell types, induction of apoptosis is associated with plasma membrane changes where PS is translocated from the inner layer of plasma membrane to the outer leaflet [33]. PS is normally restricted to the inner-membrane leaflet, however dying cells expose these phospholipids as it is one of the key signals for phagocyte recognition [34]. PS is maintained in the inner layer of the plasma membrane by the action of an ATP-dependent PS flippase. The flippase is inactivated by caspases, and a scramblase is activated, leading to redistribution of PS to the outer leaflet of plasma membrane [34]. The externalization of PS can be assessed by measuring the binding of FITC-conjugated annexin-V to cells by flow cytometry. A typical cytogram is shown where cells stained negative for both annexin-V and PI (PS-/PI-) are live cells as shown in the lower left quadrant (R3). Annexin-V positive and PI-negative (PS+/PI-) stained cells undergo early stages of apoptosis where the plasma membrane are still intact and exclude PI (lower right quadrant, R4). In late stages of apoptosis, dying cells can no longer exclude PI and the upper right region (R2) displays both annexinV-positive and PI-positive (PS+/PI+) cells. PI positive and annexinV-negative (PS-/PI+) stained cells in the upper left region (R1) are necrotic cells. Our data confirms that eurycomanone was capable of inducing cell death in HeLa cells (Figure 5A). In this experiment, the concentration of eurycomanone used for treating HeLa cells was 5 μM (IC_{50} of 3-day treatment was 5.0 ± 0.2 μM). As shown in Figure 5B, the background level of apoptosis in HeLa cells was 15.51 ± 0.28 % and the background level of necrosis was 5.64 ± 0.17 %. After exposure with eurycomanone, the level of apoptotic cells was increased to 20.9% at 24 h and 28.1% at 48 h, thus indicating that eurycomanone induced apoptosis in HeLa cells. After 48 h, there was no increasing of apoptotic level. The cells in the secondary necrosis stage were found to increase to 9.78% at 24 h, 14.71% at 48 h and 25.72% after 72 h. These results suggested that treatment of eurycomanone on HeLa

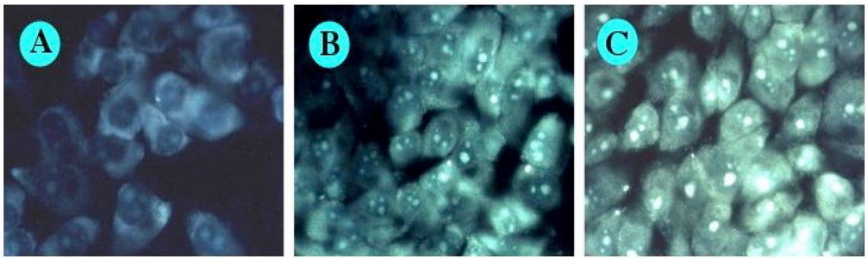

Figure 4: Nuclear staining of HeLa cells with Hoechst 33258. Cells were treated with eurycomanone compare to the untreated control. There was no fluorescence detected from untreated cells (A), while HeLa cells treated with eurycomanone showed intense fluorescence in the nuclei at 24 h (B) and even the presence of nuclear blebs at 72 h (C).

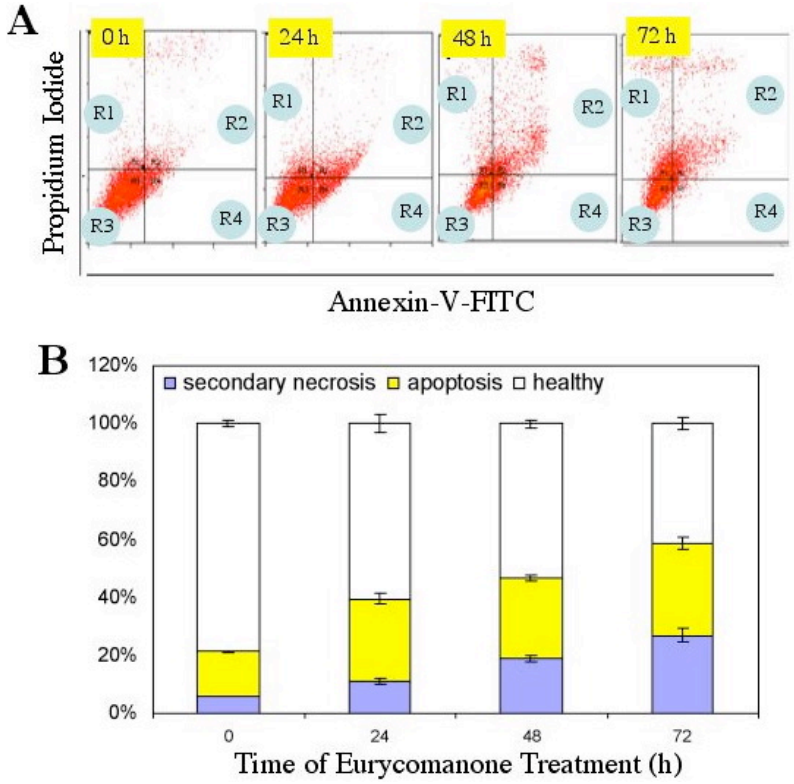

Figure 5: Flow cytometric analysis of HeLa cells. A, Flow cytograms of untreated HeLa cells and HeLa cells treated with 5 μM of eurycomanone for 24 h, 48 h and 72 h. Treatment with eurycomanone resulted a significant increase of cell death. Cells in the R1 quadrant were necrotic cells, R2 quadrant were late stage apoptotic cells, R4 quadrant were early apoptotic cells, and the cells in the R3 quadrant were live cells. B, Results were represented as means ± SEM of at least three independent experiments.

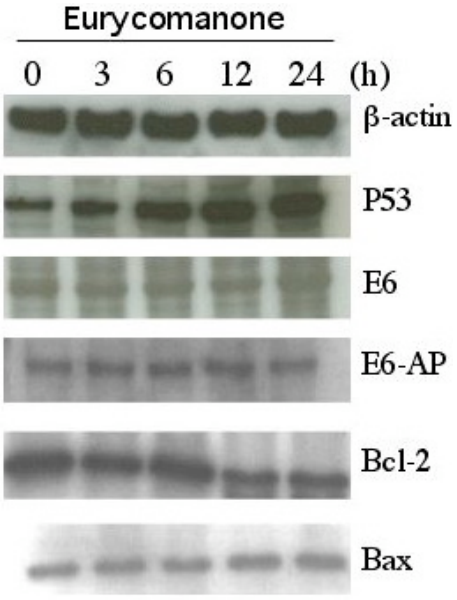

Figure 6: Western blot analysis of p53, E6, E6-AP, Bax and Bcl-2 levels in HeLa cells. HeLa cells were treated with 5 μM eurycomanone for indicated times. p53 protein expression increased after treatment and followed by increasing of Bax and decreasing of Bcl-2 level. However, E6 and E6-AP were still at basal level. β-actin was used as the internal control to confirm equal sample loading.

cells induced apoptosis and the apoptotic cells with no phagocyte surrounding them then entered to secondary necrotic stage.

Eurycomanone-induced p53 and Bax up-regulation

p53 was the first tumor suppressor gene linked to apoptosis. In most human cancers, p53 does not function correctly, and indeed, mutations in p53 have been found in nearly all tumor types and are estimated to contribute to around 50% of all cancers. p53 plays an important role in normal cell proliferation by controlling cell cycle progression and inducing apoptosis [35]. The activation of p53 can result in cell cycle arrest, presumably to allow DNA repair to occur before replication or mitosis. p53 activation can also result in apoptosis, as means of eliminating irreparable damaged cells [36]. Following eurycomanone treatment, it was found that the p53 level significantly increased. After 3 h treatment, the level of p53 was increased by 55%. The increasing level reached by 67% after 12 h and maintained at high level throughout the experiment (Figure 6). This result was confirmed by immunostaining method that demonstrated qualitatively the increasing level of p53 protein expression (Figure 7A-7C). However, E6 and E6-AP were kept at the basal levels, suggesting that eurycomanone induced apoptosis by up-regulating p53 protein without affected E6 and E6-AP in HeLa cells (Figure 6).

Bax and Bcl-2 are downstream targets of p53. In this

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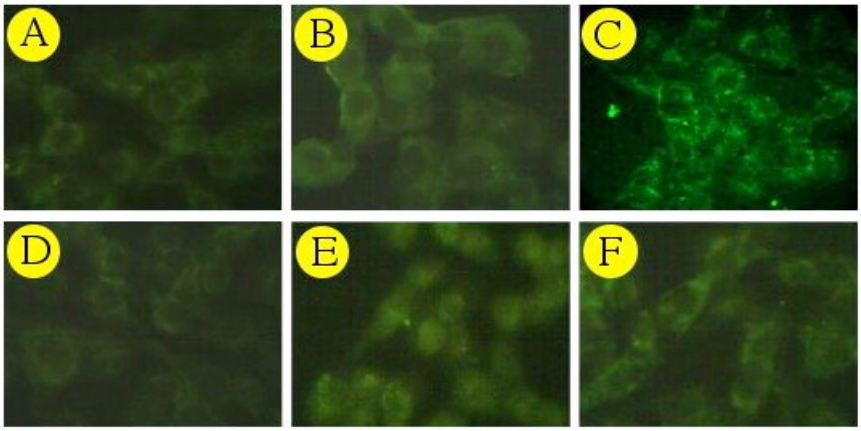

Figure 7: Immunostaining of p53 in untreated HeLa cells (A) and HeLa cells treated with 5 μM eurycomanone for 12 h (B) or 24 h (C). Immunostaining of Bax in untreated HeLa cells (D) and HeLa cells treated with 5 μM eurycomanone for 12 h (E) or 24 h (F). The intensities of immunofluorescense of both p53 and Bax were increased in eurycomanone-treated HeLa cells.

study, up-regulation of p53 was followed by the increasing of pro-apoptotic Bax protein expression and decreasing of anti-apoptotic protein Bcl-2 (Figure 6). The immunostaining assay of Bax confirmed the increasing level of Bax in eurycomanone-treated cells (Figure 7D-7F).

Discussion

Plants are a valuable source of new natural products. Despite of the availability of different approaches for the discovery of novel therapeutic agents, natural products still remain one of the best reservoirs for new molecules [37]. Natural plant products play an important role in chemotherapy, having contributed considerably to approximately 60 available cancer chemotherapeutic drugs [38]. The need to develop more effective anti-tumor drugs has prompted investigators to explore new sources of pharmacologically active compounds, especially from natural products. Eurycomanone found in *E. longifolia* is one of the novel compounds with promising potencies to be developed as a new chemotherapeutic agent. In this study, it was found that eurycomanone exerted anti-proliferative activity in HeLa cells with an IC_{50} value of 5.0 ± 0.2 μM and apoptosis was the mode of death observed in these cells. Previous study of eurycomanone on MCF-7 cells also showed the apoptotic effect [27]. This fact increases evidence that chemotherapeutic agents induce cancer cell death through the mechanism of apoptosis [39]. Additionally, eurycomanone also showed the ability to act as a cytoselective anti-cancer agent, because eurycomanone was relatively non-toxic towards non-cancerous MDBK and Vero cells. The previous study also showed the minimum effect of eurycomanone on non-cancerous breast cells (MCF-10A)[27].

Based on the result of TUNEL assay, it was found that treatment with eurycomanone induced DNA fragmentation in HeLa cells. Internucleosomal DNA fragmentation is the primary biochemical characteristic to indicate an early event of apoptosis and it represents a point of no return from the path to cell death [40]. This is due to no more new cellular protein will be synthesized for cell survival. In further analysis using Western blotting, it was found that the fundamental event that occurred when HeLa cells were treated with eurycomanone was a marked increase in the level of p53 tumor suppressor protein. The present study showed that p53 was increased 3 h after eurycomanone treatment and maintained at a higher level throughout the experiment.

p53 is an extremely efficient inhibitor of cell growth, inducing cell cycle arrest and/or apoptotic cell death, depending on cell type and environment. Therefore, regulation of p53 activity is critical to allow normal cell division. Tumor-suppressive function of p53 must be dampened sufficiently to allow normal growth and development. There are many mechanisms through which p53 is regulated. The major mechanisms include regulation of p53 protein levels, control of the localization of p53 protein and modulation of the activity of p53, particularly its ability to function as a sequence-specific transcription factor [36,41]. The incresing level of p53 in eurycomanone-treated HeLa cells could be possibly due to the increasing of p53 stability. The half-life of p53 was increased, because the p53 protein was found at an elevated level and maintained at high levels throughout the experiment. This is consistent with the previous study that p53 is a short-lived protein with a half-life of ~5-20 min [42], however, following the exposure to DNA damage, the half-life of p53 protein was increases by several folds [43]. Eurycomanone is a cytotoxic agent that might cause DNA damage in cancer cells. The DNA damages could be recognized by p53, therefore the p53 level was increased and apoptosis was induced. The previous report stated that activation of p53 could be invoked by as little as one DNA double-stranded break [44]. This DNA damage could induce dramatically increase of p53 stability as the signal could be amplified in the cells. Previous studies also reported that apoptosis in HeLa cells induced by xanthorrhizol [45], staurosporin [46], apigenin [47], vitamin C [48], carboplatin [49] and cisplatin [50] involved the increasing level of p53. The increasing level of p53 may play an important role in increasing the susceptibility of cells to undergo apoptosis. Some reports stated that loss of p53 increased chemoresistance [51-53]. The study conducted by Attardi and Jacks [54] showed that cells lacking p53 became resistant to DNA damage, failing to arrest cell cycle or undergo apoptosis. Treatment of eurycomanone resulted in increasing apoptotic cell death, which could be explained by the high level of p53.

p53 in HPV-positive cancer cells was bound by HPV E6 protein, which stimulated p53 degradation *in vitro* through the ubiquitin pathway [42]. *In vitro* analysis of the E6-p53 interaction revealed a cellular protein E6-AP that facilitated the complex formation of E6 with p53. Subsequent studies revealed that the complex of E6 and E6-AP functioned as an ubiquitin-protein ligase in the ubiquitination and subsequent degradation of p53 [56,57]. In this study, we found that the p53 level in eurycomanone-treated HeLa cells was significantly increased, however, the E6 and E6-AP levels were unchanged throughout the experiment. The effect of eurycomanone on HeLa cells was compared to xanthorrhizol which exerted cytotoxicity in HeLa cells by increasing p53 and pro-apoptotic protein Bax without any effect on E6 protein [45].

In our study, the up-regulation of p53 was followed by the increasing of pro-apoptotic protein Bax and decreasing of

anti-apoptotic protein Bcl-2 expression. p53 can transcriptionally repress the Bcl-2 expression and stimulate the Bax expression [58]. Thus, the effect of p53 on eurycomanone-induced apoptosis may be mediated, in part, through its effect on the expression of Bcl-2 and Bax. In the clinical study of cervical squamous cell carcinoma treated by radiotherapy [59], the high expression of Bax was associated with good survival, while Bcl-2 expression was associated with poor survival.

In contrast to many other human tumor forms, most HPV-positive cervical cancer cells possess wild-type p53. *p53* gene mutation in these cells was infrequent. In mutant-specific p53 analysis of cervical cancer tissues, only 12 out of 230 samples studied were found to be positive [60]. Therefore, the finding that apoptosis induced by eurycomanone through a p53-mediated pathway leads to a good track in developing eurycomanone in treatment of cervical cancer.

In conclusion, eurycomanone exerted anti-cancer activity through inducing cancer cell apoptosis. The induction of apoptosis was caused by the up-regulation of p53. However, the E6 and E6-AP was not affected. The downstream effects of the p53 up-regulation included increasing of Bax level and decreasing of Bcl-2 expression. Therefore, it was suggested that eurycomanone could be further investigated as a new alternative chemotherapeutic agent for human cervical carcinoma and other cancer that expressed the wild-type p53.

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